more preferably at least 40 % per weight, more preferably at least 50 % per weight, more preferably at least 60 % per weight, more preferably at least 70 % per weight, more preferably at least 80 % per weight, even more preferably at least 90 % per weight or up to 100 % per weight, based on the weight of the solvents involved. The preferred reaction medium is water.

According to a particularly preferred embodiment, the present invention also relates to a method and a conjugate as described above, wherein HES is reacted with the crosslinking compound, preferably with the hydroxylamino group $-O-NH_2$ of the crosslinking compound, in an aqueous medium, and wherein the reducing end of HES is not oxidized prior to this reaction.

The pH of the reaction medium the reaction of HES and the crosslinking compound is carried out in is preferably in the range of from 4.5 to 6.5, more preferably in the range of from 5.0 to 6.0 and still more preferably in the range of from 5.0 to 5.5 such as at a pH of 5.0, 5.1, 5.2, 5.3, 5.4 or 5.5.

The pH may be adjusted to the above-mentioned values with any suitably buffer such as, e.g., an acetate buffer such as a sodium acetate buffer.

Therefore, the present invention also relates to a method and a conjugate as described above, wherein the reaction of HES and the crosslinking compound is carried out at a pH of from 4.5 to 6.5.

The temperature at which this reaction is carried out is generally in the range of from 5 to 30 °C, preferably in the range of from 10 to 30 °C, more preferably in the range of from 15 to 30 °C, more preferably in the range of from 20 to 25 °C such as at a temperature of 20 °C, 21 °C, 22 °C, 23 °C, 24 °C, or 25 °C.

Therefore, the present invention also relates to a method and a conjugate as described above, wherein the reaction of HES and the crosslinking compound is carried out at a temperature of from 20 to 25 °C.

Therefore, the present invention also relates to a method and a conjugate as described above, wherein the reaction of HES and the crosslinking compound is carried out at a pH of from 5.0 to 5.5 and at a temperature of from 20 to 25 °C in an aqueous medium, and wherein the reducing end of HES is not oxidized prior to this reaction.

According to the present invention, the HES derivative resulting from the reaction of HES with the crosslinking compound is reacted with the carbohydrate moiety of EPO.

It is possible to react the HES derivative compound with the carbohydrate moiety in any suitable solvent. According to an especially preferred embodiment, this reaction is carried out in an aqueous medium.

As to the term "aqueous medium", reference is made to the definition given above.

Therefore, the present invention also relates to the method and conjugate as described above, wherein the hydroxylamino functionalized hydroxyethyl starch derivative is reacted with the carbohydrate moiety of the erythropoietin in an aqueous medium.

The pH of the reaction medium the reaction of HES derivative and the carbohydrate moiety of EPO is carried out in is preferably in the range of from 4.5 to 6.5, more preferably in the range of from 5.0 to 6.0 and still more preferably in the range of from 5.2 to 5.8 such as at a pH of 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, or 5.8, particularly preferred at about 5.5

The pH may be adjusted to the above-mentioned values with any suitably buffer such as, e.g., an acetate buffer such as a sodium acetate buffer.

Therefore, the present invention also relates to a method and a conjugate as described above, wherein the reaction of the HES derivative and the carbohydrate moiety of EPO is carried out at a pH of from 4.5 to 6.5.

The temperature at which this reaction is carried out is generally in the range of from 5 to 30 °C, preferably in the range of from 10 to 30 °C, more preferably in the range of from 15

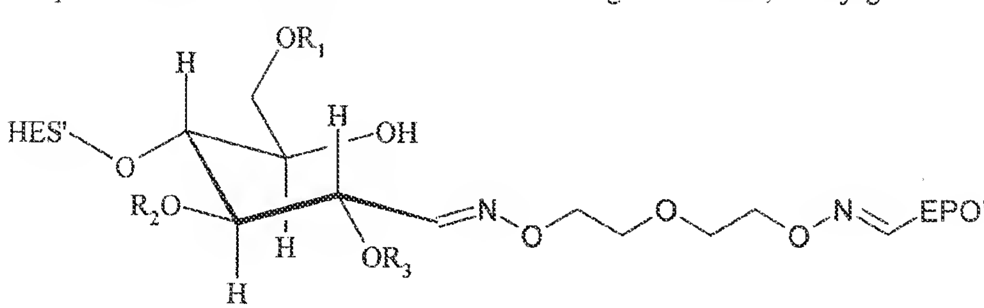
to 30 °C, more preferably in the range of from 20 to 25 °C such as at a temperature of 20 °C, 21 °C, 22 °C, 23 °C, 24 °C, or 25 °C.

Therefore, the present invention also relates to a method and a conjugate as described above, wherein the reaction of the HES derivative and the carbohydrate moiety of EPO is carried out at a temperature of from 20 to 25 °C.

Therefore, the present invention also relates to a method and a conjugate as described above, wherein the reaction of the HES derivative and the carbohydrate moiety of EPO is carried out at a pH of from 5.0 to 6.0 and at a temperature of from 20 to 25 °C in an aqueous medium.

According to an especially preferred embodiment, the present invention relates to a method wherein HES is reacted in an aqueous medium with a hydroxylamino group $-O-NH_2$ of a crosslinking compound in an aqueous medium to give a hydroxylamino functionalized HES derivative comprising an oxime linkage between HES residue and crosslinking compound residue, said method further comprising reacting the hydroxylamino functionalized HES derivative with a carbohydrate moiety of EPO, said carbohydrate moiety of EPO preferably being an oxidized terminal saccharide unit of a carbohydrate side chain of the EPO, more preferably an oxidized galactose residue and most preferably an oxidized sialic acid residue, to give a conjugate additionally comprising an oxime linkage between HES derivative residue and EPO.

Thus, according to one embodiment of the present invention where the crosslinking compound is reacted with the non-oxidized reducing end of HES, a conjugate



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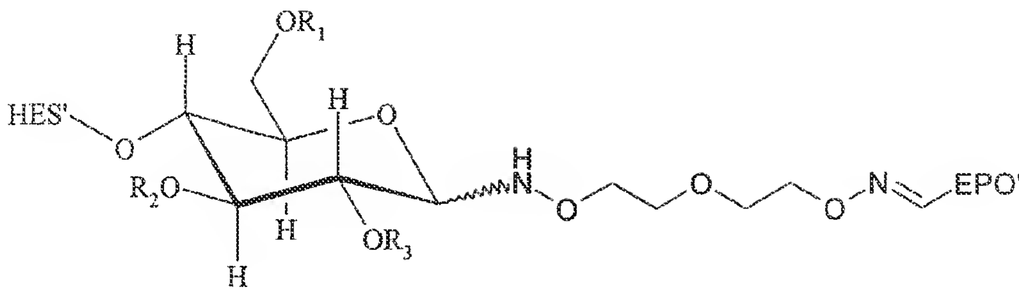

is obtained. The abbreviation EPO' refers to the EPO molecule used for the reaction without the carbon atom of the carbohydrate moiety which is part of oxime linkage in the N=C double bond. The two structures above describe a structure where the crosslinking compound is linked via an oxime linkage to the reducing end of HES where the terminal saccharide unit of HES is present in the open form, and a structure with the respective cyclic aminal form where the crosslinking compound is linked to the reducing end of HES via an oxyamino group and where the terminal saccharide unit of HES is present in the cyclic form. Both structures may be simultaneously present in equilibrium with each other.

Therefore, the present invention also relates to a conjugate comprising hydroxyethyl starch, a crosslinking compound and erythropoietin, wherein the crosslinking compound is linked via an oxime linkage and/or an oxyamino group to the hydroxyethyl starch and via an oxime linkage to the carbohydrate moiety of the erythropoietin, and wherein the hydroxyethyl starch has a mean molecular weight of at least 40 kD and a degree of substitution of at least 0.6.

According to a further aspect of the present invention, a method is provided of how to improve the in vivo activity of HES-EPO conjugates by specifically changing the characteristics of the HES used for preparing the conjugate.

Therefore, the present invention also describes a method for increasing the specific in vivo activity of a second conjugate of erythropoietin and hydroxyethyl starch compared to a first conjugate of erythropoietin and hydroxyethyl starch by using two different hydroxyethyl starches for preparing these conjugates, wherein the hydroxyethyl starch used for the preparation of the second conjugate has an increased mean molecular weight and simultaneously an increased degree of substitution DS compared to the hydroxyethyl starch used for the preparation of the first conjugate.

According to a preferred embodiment of the present invention, this method specifically applies to improving the specific in vivo activity of conjugates of erythropoietin and hydroxyethyl starch by increasing the mean molecular weight of HES from about 10 kD to at least about 40 kD, preferably to at least about 50 kD, and more preferably to about 50 kD, and simultaneously increasing the degree of substitution DS from about 0.4 to at least about 0.6, more preferably to at least about 0.7, more preferably to about 0.7 or 0.8.

Therefore, the present invention also describes a method for improving the specific in vivo activity of conjugates of erythropoietin and hydroxyethyl starch as described above, wherein the mean molecular weight is increased from about 10 kD to at least about 40 kD, preferably to about 50 kD, and the degree of substitution of the hydroxyethyl starch is increased from about 0.4 to at least about 0.6, preferably to about 0.7 to 0.8.

Therefore, e.g., in order to improve the specific in vivo activity of conjugates of erythropoietin and hydroxyethyl starch, a conjugate comprising hydroxyethyl starch having a mean molecular weight of about 10 kD and a DS of about 0.4 should be replaced by a conjugate comprising hydroxyethyl starch having, e.g., a mean molecular weight of about 50 kD and a DS of about 0.7 to about 0.8.

It is believed that this method applies to many HES-EPO conjugates, especially to HES-EPO conjugates in which HES and EPO are covalently linked via at least one crosslinking compound, particularly via one crosslinking compound which is preferably linked to HES by reacting a hydroxylamino group of the crosslinking compound with HES, most preferably in an aqueous medium, and by reacting a further hydroxylamino group of the crosslinking compound with EPO, most preferably in an aqueous medium, more preferably with a carbohydrate moiety of EPO, still more preferably with a carbohydrate moiety preferably being an oxidized terminal saccharide unit of a carbohydrate side chain of EPO such as an oxidized galactose residue or sialic acid residue.

Therefore, the present invention also relates to the method for improving the specific in vivo activity of conjugates of erythropoietin and hydroxyethyl starch as described above, wherein the conjugate comprises a crosslinking compound having two hydroxylamino groups, one of which is covalently linked to a carbohydrate moiety of the erythropoietin and one of which is covalently linked to the hydroxyethyl starch.

In the methods for preparing a conjugate of the invention the conversion rate in the above described methods may be at least 50%, more preferred at least 70%, even more preferred at least 80% and in particular 95% or even more, such as at least 98% or 99%.

Moreover, it is believed that this method for improving the specific in vivo activity of conjugates of erythropoietin and hydroxyethyl starch may also apply to other proteins and other hydroxyalkyl starches such as hydroxypropyl starches and hydroxybutyl starches. Examples of proteins are, e.g., Examples of other proteins are, e.g., colony-stimulating factors (CSF), such as G-CSF or GM-CSF like recombinant human G-CSF or GM-CSF (rhG-CSF or rhGM-CSF), alpha-Interferon (IFN alpha), beta-Interferon (IFN beta) or gamma-Interferon (IFN gamma), such as IFN alpha and IFN beta like recombinant human IFN alpha or IFN beta (rhIFN alpha or rhIFN beta), interleukines, e.g. IL-1 to IL-18 such as IL-2 or IL-3 like recombinant human IL-2 or IL-3 (rhIL-2 or rhIL-3), serum proteins such as coagulation factors II-XIII like factor VIII, alpha1-antitrypsin (A1AT), activated protein C (APC), plasminogen activators such as tissue-type plasminogen activator (tPA), such as human tissue plasminogen activator (hTPA), AT III such as recombinant human AT III (rhAT III), myoglobin, albumin such as bovine serum albumin (BSA), growth factors, such as epidermal growth factor (EGF), thrombocyte growth factor (PDGF), fibroblast growth factor (FGF), brain-derived growth factor (BDGF), nerve growth factor (NGF), B-cell growth factor (BCGF), brain-derived neurotrophic growth factor (BDNF), ciliary neurotrophic factor (CNTF), transforming growth factors such as TGF alpha or TGF beta, BMP (bone morphogenic proteins), growth hormones such as human growth hormone, tumor necrosis factors such as TNF alpha or TNF beta, somatostatine, somatotropine, somatomedines, hemoglobin, hormones or prohormones such as insulin, gonadotropin, melanocyte-stimulating hormone (alpha-MSH), triptorelin, hypthalamic hormones such as antidiuretic hormones (ADH and oxytocin as well as releasing hormones and release-inhibiting hormones, parathyroid hormone, thyroid hormones such as thyroxine, thyrotropin, thyroliberin, prolactin, calcitonin, glucagon, glucagon-like peptides (GLP-1, GLP-2 etc.), exendines such as exendin-4, leptin, vasopressin, gastrin, secretin, integrins, glycoprotein hormones (e.g. LH, FSH etc.), melanoside-stimulating hormones, lipoproteins and apo-lipoproteins such as apo-B, apo-E, apo-L_a, immunoglobulins such as IgG, IgE, IgM, IgA, IgD and fragments thereof, hirudin, tissue-pathway inhibitor, plant proteins such as lectin or ricin, bee-venom, snake-venom, immunotoxins, antigen E, alpha-proteinase inhibitor, ragweed allergen, melanin, oligolysine proteins, RGD proteins or

optionally corresponding receptors for one of these proteins; or a functional derivative or fragment of any of these proteins or receptors.

According to another aspect, the present invention also relates to method for screening for a conjugate of erythropoietin and hydroxyalkyl starch, preferably hydroxyethyl starch, having improved in vivo activity compared to native erythropoietin comprising the steps of

(i) providing a candidate conjugate;

(ii) testing the in vivo activity in comparison with native erythropoietin,

wherein the mean molecular weight MW is varied in the range of from 1 to 300 kD and the degree of substitution DS is varied in the range of from 0.1 to 1.0, and wherein these parameters are simultaneously increased compared to a given combination of parameters.

According to a preferred embodiment, the given combination of parameters is a mean molecular weight MW of about 10 kD and a degree of substitution DS of about 0.4.

Therefore, the present invention also relates to the screening method as described above, wherein the given combination of parameters is a mean molecular weight MW of about 10 kD and a degree of substitution DS of about 0.4.

According to a further aspect, the present invention also relates to the screening method as described above, said method further comprising the step of incorporating the candidate conjugate into a therapeutic or prophylactic composition.

According to yet another aspect, the present invention also relates to a conjugate as described above or a conjugate, obtainable by a method as described above, for use in a method for the treatment of the human or animal body.

The conjugates according to the invention may be at least 50% pure, even more preferred at least 70% pure, even more preferred at least 90%, in particular at least 95% or at least 99% pure. In a most preferred embodiment, the conjugates may be 100% pure, i.e. there are no other by-products present.

Therefore, according to another aspect, the present invention also relates to a composition which may comprise the conjugate(s) of the invention, wherein the amount of the

conjugate(s) may be at least 50 wt-%, even more preferred at least 70 wt-%, even more preferred at least 90 wt-%, in particular at least 95 wt.-% or at least 99 wt.-%. In a most preferred embodiment, the composition may consist of the conjugate(s), i.e. the amount of the conjugate(s) is 100 wt.-%.

Accordingly, the present invention relates to a pharmaceutical composition comprising in a therapeutically effective amount a conjugate as described above or a conjugate, obtainable by a method as described above.

The term "therapeutically effective amount" as used in the context of the present invention relates to that amount which provides therapeutic effect for a given condition and administration regimen.

Moreover, the present invention relates to a pharmaceutical composition as described above, further comprising at least one pharmaceutically acceptable diluent, adjuvant, or carrier.

According to another aspect, the present invention also relates to the use of a conjugate as described above or a HES-EPO conjugate, obtainable by a method as described, for the preparation of a medicament for the treatment of anemic disorders or hematopoietic dysfunction disorders or diseases related thereto. The invention further relates to the use of a HES-EPO conjugate as described above or a HES-EPO conjugate, obtainable by a method as described above, for the preparation of a medicament for the treatment of anemic disorders or hematopoietic dysfunction disorders or diseases related hereto.

The administration of erythropoietin isoforms is preferably by parenteral routes. The specific route chosen will depend upon the condition being treated. The administration of erythropoietin isoforms is preferably done as part of a formulation containing a suitable carrier, such as human serum albumin, a suitable diluent, such as a buffered saline solution, and/or a suitable adjuvant. The required dosage will be in amounts sufficient to raise the hematocrit of patients and will vary depending upon the severity of the condition being treated, the method of administration used and the like. The object of the treatment with the pharmaceutical composition of the invention is preferably an increase of the hemoglobin value of more than 6.8 mmol/l in the blood. For this, the pharmaceutical

composition may be administered in a way that the hemoglobin value increases between 0.6 mmol/l and 1.6 mmol/l per week. If the hemoglobin value exceeds 8.7 mmol/l, the therapy should be preferably interrupted until the hemoglobin value is below 8.1 mmol/l. The composition of the invention is preferably used in a formulation suitable for subcutaneous or intravenous or parenteral injection. For this, suitable excipients and carriers are e.g. sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chlorate, polysorbate 80, HSA and water for injection. The composition may be administered three times a week, preferably two times a week, more preferably once a week, and most preferably every two weeks. Preferably, the pharmaceutical composition is administered in an amount of 0.01-10 µg/kg body weight of the patient, more preferably 0,1 to 5 µg/kg, 0,1 to 1 µg/kg, or 0.2-0.9 µg/kg, most preferably 0.3-0.7 µg/kg, and most preferred 0.4-0.6 µg/kg body weight. In general, preferably between 10 µg and 200 µg, preferably between 15 µg and 100 µg are administered per dosis.

Accordingly, the present invention also relates to the use of a conjugate as described above or a conjugate, obtainable by a method as described above, for the preparation of a medicament for the treatment of anemic disorders or hematopoietic dysfunction disorders or diseases related thereto.

The invention is further illustrated by the following figures, tables and examples, which are in no way intended to restrict the scope of the present invention.

Short description of the Figures

Figure 1

Comparison of the Q-Sepharose eluates (HES-modified and unmodified EPO) before and after digestion with Polypeptide N-glycosidase. In each case, an aliquot corresponding to 20 µg EPO protein was applied onto the gel. The protein band at kD 21 represents the O-glycosylated EPO species without HES-modification, the diffuse band migration above represents EPO forms with HES modification at the O-glycosylation site at Ser_{126}.

Figure 2

Comparison of de-N-glycosylated EPO proteins after RP-HPLC before(-) and after (+) mild acid hydrolysis by SDS-PAGE analysis. In each case, 1.5% of the different eluates were treated with 5 mM H_2SO_4 at 83°C for 90 min or were left untreated and were applied after ethanol precipitation applied onto the gel in sample buffer.

Figure 3

Figure 3 shows an SDS page analysis of the HES—EPO conjugates, produced according to Example 3.2. For gel electrophoresis a XCell Sure Lock Mini Cell (Invitrogen GmbH, Karlsruhe, D) and a Consort E143 power supply (CONSORTnv, Turnhout, B) were employed. A 10% Bis-Tris gel together with a MOPS SDS running buffer at reducing conditions (both Invitrogen GmbH, Karlsruhe, D) were used according to the manufactures instruction.

Lane A: Protein marker SeeBlue®Plus2 (Invitrogen GmbH, Karlsruhe, D) Molecular weight marker from top to bottom: 188 kD, 98 kD, 62 kD, 49 kD, 38 kD, 28 kD, 17 kD, 14 kD, 6 kD, 3 kD

Lane B: Crude reaction product Example 3.2(a)

Lane C: Crude reaction product Example 3.2(b)

Lane E: Crude reaction product Example 3.2(d)

Lane F: Crude reaction product Example 3.2(c)

Lane G: Oxidized EPO according to Example 2.

Figure 4

HPAEC-PAD analysis of native N-linked oligosaccharides from untreated, periodate oxidised and HES-modified EPO.

1 represents total oligosaccharides from the EPO starting material (A14),
2 represents oligosaccharides obtained from the Q-sepharose purified product modified with HES 10/0.4 (A20),
3 represents oligosaccharides obtained from the Q-sepharose purified product modified with HES 10/0.7 (A21),
5 represents oligosaccharides obtained from the Q-sepharose purified product modified with HES 50/0.7 (A23),
6 represents oligosaccharides obtained from the Q-sepharose purified periodate treated EPO (A24),
7 represents oligosaccharides obtained from the Q-sepharose purified product modified with HES 50/0.4 (A25).

0 represents the neutral oligosaccharide fraction,
I represents the mono-charged oligosaccharide fraction (1 sialic acid),
II represents the di-charged oligosaccharide fraction (2 sialic acid),
III represents the tri-charged oligosaccharide fraction (3 sialic acid),
IV represents the tetra-charged oligosaccharide fraction (4 sialic acid).

Figure 5

HPAEC-PAD analysis of N-linked oligosaccharides after mild acid treatment from untreated, periodate oxidised and HES-modified EPO (see Example 6.3(b)).

1 represents N-acetylneuraminic acid
2 represents a diantennary structure + alpha1-6 linked fucose

3 represents a triantennary structure + alpha 1-6 linked fucose

4 represents a triantennary structure (isomer), + alpha 1-6 linked fucose

5 represents a tetraantennary structure, + alpha 1-6 linked fucose

6 represents a tetraantennary structure plus 1 N-acetyllactosamin repeat + alpha 1-6 linked fucose

7 represents a tetraantennary structure plus 2 N-acetyllactosamin repeat + alpha 1-6 linked fucose.

0 represents oligosaccharides after mild acid hydrolysis from EPO starting material (A14),

I represents oligosaccharides after mild acid hydrolysis from EPOmodified with HES 10/0.4 (A20),

II represents oligosaccharides after mild acid hydrolysis from EPO modified with HES 10/0.7 (A21),

IV represents oligosaccharides after mild acid hydrolysis from EPO modified with HES 50/0.7 (A23),

V represents oligosaccharides after mild acid hydrolysis from EPO modified by mild periodate oxidation (A24),

VI represents oligosaccharides after mild acid hydrolysis from EPO modified with HES 50/0.4 (A25),

Examples

Sample code HES-modification of EPO

0312-17/**A14**	EPO starting material, not modified
0401-09/**A20**	HES 10/0.4; periodate oxidised
0401-09/**A21**	HES 10/0.7 ; periodate oxidised
0401-09/**A23**	HES 50/0.7 ; periodate oxidised
0401-09/**A24**	A14/mock-incubated with unmodified HES; periodate oxidised
0401-09/**A25**	HES 50/0.4 ; periodate oxidised

Sample identification (short form) e.g. for the starting material is **A14; A20** = periodate oxidised EPO A14 after modification with HES 10/04.

Example 1: Periodate oxidation of N-acetylaneuraminic acid residues by mild periodate treatment of EPO

To a 2,0 mg/ml solution of EPO (recombinantly produced EPO having amino acid sequence of human EPO and similar or essentially the same characteristics as the commercially available Epoietin alpha :Erypo, ORTHO BIOTECH, Jansen-Cilag or Epoietin beta: NeoRecormon, Roche; cf. EP 0 148 605, EP 0 205 564, EP 0 411 678) of total 20ml kept at 0°C were added 2,2ml of an ice-cold solution of 10mM sodium meta-periodate resulting in a final concentration of 1mM sodium meta-periodate. The mixture was incubated at 0°C for 1 hour in an ice-bath in the dark and the reaction was terminated by addition of 40µl of glycerol and incubated for further 5 minutes.

Example 2: Buffer exchange of periodate oxidised EPO for subsequent derivatisation with a hydroxylamino functionalized hydroxyethyl starch derivative

Buffer exchange was performed using a 20 ml Vivaspin 20 concentrator (Vivaspin AG, Hannover, Germany) with a polyethersulfone (PES) membrane and a molecular weight

cut-off 10 kD. First, the concentrator unit was washed by addition of 5 ml of 0.1 M Na-acetate buffer pH 5.5 and centrifugation of the concentrator unit at 4000 rpm at 6°C in a Megafuge 1.0R (Kendro Laboratory Equipment, Osterode, Germany). Subsequently, 20 ml of the periodate oxidised EPO solution according to Example 1 was added to the concentrator unit and was centrifuged at 4000 rpm for 25min until a 5-fold concentration was achieved. 15 ml of 0.1 M Na-acetate buffer pH 5.5 was added to the concentrate and then centrifuged as described above. The centrifugation cycle was repeated 3 times, the final concentrate was removed and transferred into a 50 ml sterile plastic tube, after washing of the concentrator unit 2 times with each 1 ml of Na-acetate buffer pH 5.5; the volume of the EPO solution was adjusted with Na-acetate buffer pH 5.5 to 26.7 ml and protein concentration of the final oxidised EPO solution was determined by measuring the absorbance at 280 nm using the specific absorbance value of 7.43 as described in the European Pharmacopeia (Erythropoietin Concentrated Solution, 4th Edition, 2002, pages 1123-1128). A value of 1.378 mg / ml was determined for the final periodate oxidised EPO solution (36.8 mg EPO, corresponding to ≈ 90 % final yield).

Example 3 Synthesis of conjugates of hydroxyethyl starch and EPO

Example 3.1 Synthesis of hydroxylamino functionalized HES derivatives

O-[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine was synthesized as described in Boturyn et al. Tetrahedron 53 (1997) p. 5485-5492 in 2 steps from commercially available materials.

Example 3.1(a) Synthesis of hydroxylamino-HES 10 / 0.4

2 g of HES10/0.4 (MW = 10000 D, DS = 0.4, Supramol Parenteral Colloids GmbH, Rosbach-Rodheim, D) were dissolved in 17 mL 0.1M sodium acetate buffer, pH 5.2 and 20 mmol O-[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine were added. After shaking for 19 h at 22°C, the reaction mixture was added to 100 mL of an ice-cold 1:1 mixture of acetone and ethanol (v/v). The precipitated product was collected by centrifugation at 4°C, re-dissolved in 50 mL water, dialysed for 21 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized.

The molecular weight of the HES10/0.4 when measured with LALLS-GPC was 8.4 kD and the DS was 0.41.

Example 3.1(b) Synthesis of hydroxylamino-HES 10 / 0.7

2 g of HES10/0.7 (MW = 10000 D, DS = 0.7, Supramol Parenteral Colloids GmbH, Rosbach-Rodheim, D) were dissolved in 18 mL 0.1M sodium acetate buffer, pH 5.2 and 20 mmol O-[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine were added. After shaking for 19 h at 22°C, the reaction mixture was added to 100 mL of an ice-cold 1:1 mixture of acetone and ethanol (v/v). The precipitated product was collected by centrifugation at 4°C, re-dissolved in 50 mL water, dialysed for 21 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized.

The molecular weight of the HES10/0.7 when measured with LALLS-GPC was 10.5 kD and the DS was 0.76.

Example 3.1(c) Synthesis of hydroxylamino-HES 50 / 0.4

2 g of HES50/0.4 (MW = 50000 D, DS = 0.4, Supramol Parenteral Colloids GmbH, Rosbach-Rodheim, D) were dissolved in 20 mL 0.1M sodium acetate buffer, pH 5.2 and 4 mmol O-[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine were added. After shaking for 19 h at 22°C, the reaction mixture was added to 100 mL of an ice-cold 1:1 mixture of acetone and ethanol (v/v). The precipitated product was collected by centrifugation at 4°C, re-dissolved in 50 mL water, dialysed for 21 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized.

The molecular weight of the HES50/0.4 when measured with LALLS-GPC was 55.7 kD and the DS was 0.41.

Example 3.1(d) Synthesis of hydroxylamino-HES 50 / 0.7

2 g of HES50/0.7 (MW = 50000 D, DS = 0.7, Supramol Parenteral Colloids GmbH, Rosbach-Rodheim, D) were dissolved in 20 mL 0.1M sodium acetate buffer, pH 5.2 and 4 mmol O-[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine were added. After shaking for

17.5 h at 22°C, the reaction mixture was added to 70 mL of an ice-cold 1:1 mixture of acetone and ethanol (v/v). The precipitated product was collected by centrifugation at 0°C, washed with 30 mL of an ice-cold 1:1 mixture of acetone and ethanol (v/v), re-dissolved in 50 mL water, dialysed for 19.5 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized.

The molecular weight of the HES50/0.7 when measured with LALLS-GPC was 46.9 kD and the DS was 0.76.

Example 3.2 Synthesis of HES-EPO conjugates

In Examples 3.2(a) to 3.2(d), a successful conjugation is indicated by the migration of the protein bands to higher molecular weights in the SDS page analysis according to Figure 3. The increased band-with is due to the molecular weight distribution of the HES derivatives used and the number of HES derivatives linked to the protein.

Example 3.2(a) Synthesis with of hydroxylamino-HES 10 / 0.4 according to Example 3.1(a)

To 3.63 mL of a solution of oxidized EPO in 0.1 M sodium acetate buffer, pH 5.5 (according to Example 2; 1.378 mg/ml), 83 mg of hydroxylaminoHES10/0.4, produced according to example 3.1(a), were added and the solution was shaken for 16.5 h at 22°C.

Example 3.2(b) Synthesis with of hydroxylamino-HES 10 / 0.7 according to Example 3.1(b)

To 3.63 mL of a solution of oxidized EPO in 0.1 M sodium acetate buffer, pH 5.5 (according to Example 2; 1.378 mg/ml), 83 mg of hydroxylaminoHES10/0.7, produced according to example 3.1(b), were added and the solution was shaken for 16.5 h at 22°C.

Example 3.2(c) Synthesis with of hydroxylamino-HES 50 / 0.4 according to Example 3.1(c)

To 3.63 mL of a solution of oxidized EPO in 0.1 M sodium acetate buffer, pH 5.5 (according to Example 2; 1.378 mg/ml), 416 mg of hydroxylaminoHES50/0.4, produced according to example 3.1(c), were added and the solution was shaken for 16.5 h at 22°C.

Example 3.2(d) Synthesis with of hydroxylamino-HES 50 / 0.7 according to Example 3.1(d)

To 3.63 mL of a solution of oxidized EPO in 0.1 M sodium acetate buffer, pH 5.5 (according to Example 2; 1.378 mg/ml), 416 mg of hydroxylaminoHES50/0.7, produced according to example 3.1(d), were added and the solution was shaken for 16.5 h at 22°C.

Example 4 Purification of HES-modified EPO and separation of unreacted HES-derivatives from HES-modified EPO

Subsequent to the HES-coupling procedures according to Examples 3.2(a) to 3.2(d), the purification of all samples was performed at room temperature using an ÄKTA explorer 10 system equipped with a Pump P-903, Mixer M-925 with 0.6 ml chamber, Monitor pH/C-900, pump P-950 (sample pump) along with a Software Unicorn Version 3.21. Detection was at 280, 260 and 220 nm using a Monitor UV-900 with a 10 mm flow cell.

The incubation mixtures were diluted with 10 volumes of buffer A (20 mM N-morpholino propane sulfonic acid adjusted to pH 8.0 with NaOH) and were applied to a column containing 4 ml Q-Sepharose Fast Flow (Amersham Pharmacia Biotech) at a flow rate of 0.8 ml/min; the column was previously equilibrated with 7 column volumes (CV) of buffer A. The column was then washed with 6 CV of buffer A at a flow rate of 1.0 ml/min and elution was performed by using 2.5 CV of buffer B (0.5 M NaCl in 20 mM Na-phosphate, pH 6.5) at a flow rate of 0.6 ml/min. The column was then washed with 2.5 CV of buffer C (1.5 M NaCl in 20 mM Na-phosphate, pH 6.5) at a flow rate of 0.6 ml/min and was re-equilibrated by passing 7 CV of buffer A at flow rate of 1.0 ml/min.

Samples from incubations with (activated) hydroxylaminoHES derivatives all yielded significant absorption at 220 nm. Samples A20 and A21 (incubated with hydroxylaminoHES10/0.4 and 10/0.7, respectively) gave no detectable absorption at 280 nm, whereas samples A23 and A25 (incubated with HydroxylaminoHES50/0.7 and 50/0.4, respectively) yielded 800 mAU x ml and 950 mAU x ml, respectively. The bound proteins were recovered in a volume of 6.5 - 8.0 ml almost exclusively in eluate 1, with eluate 2 containing < 2% of the peak area of totally eluted peaks detected at 280 nm. The protein recovery was comparable for all EPO samples (approximately 85%).

HES-modified EPO and EPO from appropriate control incubations were subjected to buffer exchange by using 5 ml Vivaspin concentrators (10,000 MW cut-off) and centrifugation at 4000 rpm at 6°C as described previously. Samples (1-3 mg of EPO protein) were concentrated to 0.5-0.7 ml and were diluted with phosphate buffered saline (PBS) pH 7.1 to 5 ml and subjected to 10-fold concentration by centrifugation. Each sample was subjected to the concentration and dilution cycle three times. Finally, samples were withdrawn and the concentrator units were washed with 2x 0.5 ml of PBS. Samples were frozen in liquid nitrogen at protein concentrations of approximately 1.2 mg/ml.

Example 5 Analytical experiments

Example 5.1 Liberation of N-linked oligosaccharides with recombinant polypeptide N-glycosidase (Roche, Penzberg, Germany)

To 400 – 1.2 mg aliquots of native, periodated oxidised or HES-modified EPO in 50 mM Na-phosphate buffer pH 7.2 were added 40µl of recombinant polypeptide N-glycosidase (Roche, Penzberg, Germany; 250 units/250 µl lot: 101610420). The reaction mixture was incubated at 37°C for 12 - 18 hours and the release of N-glycosidically bound oligosaccharides was checked by SDS-PAGE analysis of 5-10µg protein under reducing conditions and subsequent staining of protein bands with Coomassie Blue (Carl Roth GmbH Karlsruhe, Germany) and detection of the specific shift of the EPO protein band to the migration position of the de-N-glycosylated EPO forms.

Example 5.2 Separation of N-linked oligosaccharides from de-N-glycosylated EPO protein by RP-HPLC

Separation of all de-N-glycosylated EPO samples from HES-modified and unmodified EPO protein samples was performed at room temperature using an ÄKTA explorer 10 system equipped with a Pump P-903, Mixer M-925 with 0.6 ml chamber, Monitor pH/C-900, pump P-950 (sample pump) along with a Software Unicorn Version 3.21. Detection was at 280, 220 and 206 nm using a Monitor UV-900 with a 10 mm flow cell.

Runs were performed at room temperature using the ÄKTA explorer 10 equipment and flow rate of 4 ml/min. Aliquots of PNGase digests of 1.1-1.2 mg HESylated EPO were applied to a PepRPC 15 µm column (PepRPC 15µm, 2 cm x 10 cm; Pharmacia) which was equilibrated with 1.25 CV of 9% eluent B (0.1 % TFA, 90% acetonitrile). 1.25 ml samples of de-N-glycosylated EPO forms were then injected and the sample loop was washed with 12 ml of 9% eluent B. Following washing of the column with 0.2 CV of 9% eluent B, a linear gradient from 9% to 90% eluent B over 2 CV was applied. Elution of the column was continued by using 0.5 CV of 90% eluent B, and finally the column was re-equilibrated with 1.0 CV of 9% eluent B. Fractions were collected every 1 min (4 ml).

The oligosaccharides were recovered from the flow through (fractions 1-3; 4 ml each fraction) and, in the case of HESylated EPO, from fractions 6-8 eluting at a concentration of about 20% eluent B. The protein eluted in a volume of 10-12 ml at a concentration of 54% eluent B. The recovery of the de-N-glycosylated EPO was comparable for all samples, yielding a mean value of 581 mAU x ml x mg^{-1}, with a relative standard deviation of 3.8%.

(a) The EPO protein fractions (containing EPO forms modified with HES at the O-glycan moiety) were diluted with 1 volume of water and were lyophilized. Subsequently the dried samples were re-solubilized in water and after neutralisation with NaOH were subjected to concentration using 5 ml Vivaspin concentrators.

(b) The oligosaccharide fractions (see above : fractions 1-3 combined with fractions 6-8, respectively) were concentrated in a speed-vac concentrator after neutralisation and were subsequently de-salted using Vivaspin 5 concentrators (cut-off 5000). The oligosaccharides

were adjusted to a final volume of 0.5 ml and were frozen in liquid nitrogen and kept at -20°C.

(c) For analytical purposes, the released N-glycans were separated from the polypeptide by addition of 3 volumes of cold 100 % ethanol and incubation at –20°C for at least 2 hours. The precipitated protein was removed by centrifugation at 13,000 rpm for 10 minutes at 4°C. The pellet was then subjected to two additional washes with 500 µl ice-cold 70% ethanol. The oligosaccharides in the pooled supernatants were dried in a vacuum centrifuge (Speed Vac concentrator, Savant Instruments Inc., USA). The glycan samples were desalted using Hypercarb cartridges (100 or 200 mg) as follows: prior to use, the cartridges were washed three times with 500 µl 80% (v/v) acetonitrile in 0.1% (v/v) TFA followed by three washes with 500 µl water. The samples were diluted with water to a final volume of at least 300 µl before loading onto the cartridges. They were rigorously washed with water.

Oligosaccharides were eluted with 1.2 ml 25% acetonitrile containing 0.1% (v/v) TFA. The eluted oligosaccharides were neutralised with 2 M NH_4OH and were dried in a Speed Vac concentrator. They were stored at -20°C in H_2O until further use.

Example 5.3 Oligosaccharid analysis - Mild acid hydrolysis of oligosaccharides (removal of sialic acids and HES-modified sialic acids from oligosacharides)

Aliquots of the desalted oligosaccharides (compare 5.2(a)) were mixed with the same volume of 10mM H_2SO_4 and were incubated for 90 minutes at 80°C. After neutralisation with 50 mM NaOH the desialylated glycans were dried in a speed-vac and were adjusted to an appropriate concentration for analysis in HPAEC-PAD (high-pH-anion exchange chromatography with pulsed amperometric detection).